ECDYSTEROIDS IN DEVELOPING OVARIES AND EGGS OF THE TOBACCO HORNWORM

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ABSTRACT

Ecdysteroids of ovaries and newly-laid eggs (0- to 1-hour-old) of the tobacco hornworm are present mainly as conjugates (>95%). Newly-laid eggs contain ecdysteroid conjugates equivalent to 21 μg of 26-hydroxyecdysone and 0.73 μg of ecdysone per gram of eggs. These levels are similar in ovaries of 93-hour-old adult females. In 1- to 18-hour-old eggs more than 63% of the ecdysteroids exist in the free form and the proportion is similar in 48- to 64-hour-old eggs. The ratio of 26-hydroxyecdysone to ecdysone in the conjugated form remains constant during oocyte maturation and embryogenesis. Though 26-hydroxyecdysone is without molting hormone activity in the house fly assay, the exceptionally high concentration of 26-hydroxyecdysone conjugate(s) in ovaries and newly-laid eggs, together with the fact that it is being released during embryogenesis, indicate some physiological role for 26-hydroxyecdysone.

INTRODUCTION

The free ecdysteroids from three different age groups of embryonated eggs of the tobacco hornworm, Manduca sexta (L.), 48- to 64-hour, 24- to 44-hour, and 4- to 18-hour-old, were reported in previous communications from this laboratory [1,2,3]. From extracts of kilogram quantities of eggs of these three age groups, milligram quantities of ecdysteroids were isolated [1,2,3]. 26-Hydroxyecdysone (which accounted for 80 to 90 percent of the ecdysteroids), ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone have been identified in all three age groups. The 4- to 18-hour old eggs also contained low levels of 3-epi-26-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone as well as six other unidentified ecdysteroids which lacked molting hormone activity [3]. Interestingly, 26-hydroxyecdysone is devoid of molting hormone activity in the house fly bioassay and has been

reported at such high levels only in eggs of the tobacco hornworm. The ecdysteroid composition of tobacco hornworm eggs suggested that there were at least two biosynthetic pathways for ecdysteroids during embryonic development of the hornworm: the pathway to 26-hydroxyecdysone as the principal route and the formation of 20-hydroxyecdysone as a minor pathway [4], and ecdysone could serve as an intermediate in both pathways. Since studies of other insect species [5,6,7] have shown that an impressively large quantity of the ecdysteroids in newly-laid eggs and ovaries existed in the conjugated form, we investigated the possibility that ecdysone, the putative precursor of 26-hydroxyecdysone, could be stored in the form of conjugates in ovaries and newly-laid eggs of the tobacco hornworm. This report is concerned with the titer of free and conjugated ecdysteroids of various developmental stages of ovaries and embryonated hornworm eggs.

MATERIALS AND METHODS

Biological Material: Tobacco hornworms were reared as described previously [8]. Ovaries were excised from adult females 24 or 93 hours after eclosion and samples of each age group were held frozen in methanol at -20° until at least 10 g were accumulated for extraction. Females would ordinarily begin laying eggs the night following the 93-hour time period. Eggs, 0- to 1-hour-old, and 1- to 18-hour-old (mostly 18-hr old), were collected at the indicated times from leaves of the tobacco plants provided for egg deposition. Eggs for the 48- to 64-hour samples were removed from the tobacco plant at approximately 18 hr and kept in petri dishes for the remainder of the time interval [1]. The eggs were weighed, transferred into screw cap glass bottles and held frozen (-20°) until several 10-g lots were accumulated for workup.

Enzymes: β-Glucuronidase Type H-1 from Helix, β-glucuronidase Type L-II from limpets (both also contain sulfatase and phosphatase activity), and β-D-glucosidase from almonds were all purchased from Sigma [9].

Extraction and Separation of Free and Conjugated Ecdysteroids: The ovary or egg samples (10 g) in 40 ml of methanol were homogenized with a Polytron homogenizer (Brinkmann Instruments). The homogenates were

centrifuged and the supernatants were pipetted off. The residues were rehomogenized in 40 ml of 70% methanol and the supernatants were collected as before. This step was repeated. Combined supernatants were reduced to dryness under vacuum below 40°. To remove the apolar lipids, the residue (450-500 mg) was partitioned in a centrifuge tube between 15 ml each of pre-equilibrated hexane and 70% methanol. Two additional centrifuge tubes, each containing 15 ml of the lower phase, were used in the partitioning and 3 transfers of the upper phase were made (if emulsions were formed, tubes were centrifuged to separate the phases). The upper phases were discarded. The 70% methanol phases were combined and reduced to dryness under vacuum. The residue was then partitioned between 6 ml each of pre-equilibrated butanol and water over three centrifuge tubes. Five transfers of the upper phase over the three tubes were made. The combined lower phases, containing the ecdysteroid conjugates, were set aside for enzymatic hydrolysis. The combined upper phases, containing the free ecdysteroids, were reduced to dryness under vacuum below 40° and the residue (19-24 mg) was purified and analyzed by column chromatography.

Enzymatic Hydrolysis of Conjugates and Isolation of Ecdysteroids: The conjugate fractions obtained from ovaries or eggs were reduced to dryness under vacuum at 40°. The residues (350-400 mg) of individual samples were dissolved in 9 ml of 0.2 M sodium acetate-acetic acid buffer solution (pH 5). Three ml of a freshly prepared solution of 7 mg of β-glucuronidase (Type H-1) and 4 mg each of β-glucuronidase (Type L-II) and β-D-glucosidase in 0.2 M sodium chloride were added to each. The mixtures were incubated at 30° for 48 hr, then 3 ml each of methanol and butanol were added and the mixtures were reduced to a volume of 0.5 ml and partitioned between 6 ml each of pre-equilibrated butanol and water over 3 tubes. Five transfers were made and the upper phases were combined and reduced to dryness under vacuum to give 2.5-4 mg of residue that now contained the hydrolyzed ecdysteroids.

Chromatography of Free Ecdysteroids and Ecdysteroids from Conjugates: The residues, which contained the free ecdysteroids or ecdysteroids from conjugates, were dissolved in 250 µl of methanol, then diluted to 5 ml with chloroform. Each solution was placed on a column of chloroform-washed Unisil or E. Merck silica gel (4g, 1 x 13 cm). The columns were eluted with 40-ml volumes of each of the following: 1) chloroform-5% ethanol, 2) chloroform-7.5% ethanol, 3) chloroform-10% ethanol, 4) chloroform-15% ethanol, 5) chloroform-20% ethanol, 6) chloroform-30% ethanol, 7) chloroform-40% ethanol, and 8) chloroform-50% ethanol. All fractions were monitored by TLC (HP-TLC pre-coated plates Silica Gel 60F 254 for nano TLC, E. Merck). Fractions 4, combined fractions 5-6, and fraction 7 were each analyzed by reversed-phase HPLC (RP-HPLC) on C_{18} µ-Bondapak (Waters Associates).

RP-HPLC Analysis: RP-HPLC analyses of ecdysteroid fractions were performed on a C_{18} µBondapak (4.0 mm x 30 cm) column at 40° by isocratic elution with a 35% methanol/water mixture at a flow rate of 0.8 ml/min. Absorbance of the effluent was monitored at 254 nm with a Waters Model 441 absorbance detector and automatically recorded by a

Shimadzu Model C-RIB recording integrator. 20-Hydroxyecdysone, 26-hydroxyecdysone, and ecdysone eluted at 8.24, 9.54, and 14.14 min, respectively. Ecdysteroids in the chromatographic fractions were quantified by comparison of the peak areas with calibrated areas obtained with known amounts of the authentic ecdysteroids.

RESULTS AND DISCUSSION

The butanol-water partitioning system, as employed, effectively separates the free ecdysteroids and ecdysteroid conjugates of ovaries and eggs of the tobacco hornworm. In fact, preliminary studies indicated that, when extracts of 0- to 1-hour-old eggs were partitioned in the butanol-water system over 4 tubes through 7 transfers of the upper phase, tube 1 contained the greatest quantity of conjugates followed by tube 2, and only a small quantity was found in tubes 3 and 4. Thus, the approximate partition coefficients of 7 and 5.5 for 26-hydroxyecdysone and 20-hydroxyecdysone, respectively [10] in the butanol-water system and the negligible carry-over of the ecdysteroid conjugates with the butanol transfers indicate that a complete extraction of the free ecdysteroids into the butanol phase without any appreciable quantity of conjugates is possible.

The ecdysteroids in the butanol phase can be further purified by partitioning into the upper phase of the solvent system cyclohexane-butanol-water (5:5:10). The low distribution coefficient of 0.39 for 26-hydroxyecdysone in this system [1], however, makes it necessary to conduct a large number of transfers with the upper phase (>12) over 3 tubes in order to obtain a complete extraction. Column chromatography of the butanol phase over Unisil purifies the ecdysteroids sufficiently so that they can be analyzed on Nano TLC plates as well as quantified by HPLC. Ecdysone was eluted in fraction 4, and 26-hydroxyecdysone in fractions 5 and 6 and the latter two

Table 1

Titer of free and conjugated ecdysteroids of ovaries and eggs at various developmental stages of the tobacco hornworm

	Free ecdysteroids µg/g fresh weight*	Conjugated Ecdysteroids µg/g fresh weight*†	Sum of free and conjugated ecdysteroids
Ovaries from 24-hour-old adult females			
Ecdysone	not detected	0.25	0.25
26-Hydroxyecdysone	0.65	8.70	9.35
Ovaries from 93-hour-old adult females			
Ecdysone	not detected	0.58	0.58
26-Hydroxyecdysone	not detected	20.20	20.20
0- to 1-hour-old eggs			
Ecdysone	not detected	0.73	0.73
26-Hydroxyecdysone	0.96	21.00	21.96
1- to 18-hour-old eggs			
Ecdysone	0.38	0.22	0.60
26-Hydroxyecdysone	13.40	7.80	21.20
48- to 64-hour-old eggs**			
Ecdysone	0.02	not detected	0.02
26-Hydroxyecdysone	6.90	6.0	12.90

*Determined by RP-HPLC
**These eggs also showed 20-hydroxyecdysone (0.03 µg/g) in the free ecdysteroid fraction.
†Expressed as free ecdysteroids recovered after enzymatic hydrolysis of conjugates.

fractions were combined for HPLC analysis.

Our results (Table 1) show that the ecdysteroids of ovaries and newly-laid eggs of the tobacco hornworm are present mainly as conjugates (>95%). This has also been shown for certain other insect

species [5,6,7], however, unlike conjugates of other insect species, the conjugates of the hornworm yield primarily 26-hydroxyecdysone and only very small amounts of ecdysone after enzymatic hydrolysis. The levels of ecdysteroids recovered from the conjugates of newly-laid eggs are 21 µg of 26-hydroxyecdysone and 0.73 µg of ecdysone per gram of eggs (Table 1). The level of free 26-hydroxyecdysone found in newly-laid eggs was 0.96 µg/g. Ecdysone was not detected. During embryogenesis, the ecdysteroid conjugates are hydrolyzed and in 1- to 18-hour-old eggs more than 63% of the ecdysteroids exist in the free form (Table 1). Surprisingly, the results were similar in 48- to 64-hour-old eggs. The levels of free 26-hydroxyecdysone in 1- to 18-hour-old and 48- to 64-hour-old eggs, 13.4 µg and 6.9 µg/g, respectively, compare well with the levels found previously (12 and 5.3 µg/g) in similar age groups [1,3].

Although the ratio of 26-hydroxyecdysone to ecdysone in the conjugated form in ovaries of 24-hour-old females (35:1) is identical to that found in ovaries of 93-hour-old females (35:1) and similar to that of 0- to 1-hour-old eggs (29:1), the total quantity of both ecdysteroids (µg/g of tissue) in 24-hour ovaries is less than half of that found in 93-hour ovaries or 0- to 1-hour-old eggs. The highest ecdysone content was found in the conjugates of 0- to 1-hour-old eggs (0.73 µg/g of tissue). Our results do not implicate ecdysone as a precursor of 26-hydroxyecdysone in the eggs. The answer to the question whether ecdysone is a precursor for 26-hydroxyecdysone in ovaries and eggs of the tobacco hornworm will have to await the *in vivo* test for the conversion of labeled ecdysone to 26-hydroxyecdysone.

Our qualitative and quantitative analyses of the ecdysteroid conjugates were carried out after enzymatic hydrolysis. Thus, the detection of the conjugates depended on the ability of the enzymes employed to hydrolyze these conjugates. Although the hydrolyses yielded substantial amounts of free ecdysteroids, it cannot be ruled out that some conjugates remained unhydrolyzed after the enzyme incubations and were not detected in the present study.

Certainly, the preponderance of conjugated 26-hydroxyecdysone in ovaries and newly-laid eggs of the tobacco hornworm, together with the fact that the conjugates are being hydrolyzed during embryogenesis indicate a physiological role for 26-hydroxyecdysone other than serving as an inactivation product. However, a considerable amount of research remains to be done before we can begin to determine or understand the role of ecdysone and 26-hydroxyecdysone or their conjugates as well as their metabolic fate in developing embryos. It is important that the ecdysteroids of first instar larvae be identified, that the structures of the six other previously reported unidentified ecdysteroids of eggs be determined, and that the ecdysteroid conjugates be identified. We have already initiated research in certain of these areas. Since 26-hydroxyecdysone is a possible precursor for ecdysonoic acid, a metabolite found in developing eggs of Schistocerca gregaria, pupae of Spodoptera littoralis [11], and larvae and pupae of Pieris brassicae [12], a search for this compound in tobacco hornworm ovaries and eggs is also being conducted.

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9. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.
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TRIVIAL AND IUPAC EQUIVALENT NAMES

Ecdysone = 2β,3β,14α,22R,25-Pentahydroxy-5β-cholest-7-en-6-one

20-Hydroxyecdysone = 2β,3β,14α,20R,22R,25-Hexahydroxy-5β-cholest-7-en-6-one

26-Hydroxyecdysone = 2β,3β,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one

20,26-Dihydroxyecdysone = 2β,3β,14α,20R,22R,25,26-Heptahydroxy-5β-cholest-7-en-6-one

3-Epi-20,26-dihydroxyecdysone = 2β,3α,14α,20R,22R,25,26-Heptahydroxy-5β-cholest-7-en-6-one

3-Epi-26-hydroxyecdysone = 2β,3α,14α,22R,25,26-Hexahydroxy-5β-cholest-7-en-6-one

Ecdysonoic acid = 2β,3β,14α,22R,25-pentahydroxy-6-oxo-5β-cholest-7-en-26-oic acid